Reverse Structural and Gap-Junctional Remodeling After Prolonged Atrial Fibrillation in the Goat

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Background—Prolonged atrial fibrillation (AF) results in electrical, structural, and gap-junctional remodeling. We examined the reversibility of the changes in (ultra)structure and gap junctions.

Methods and Results—Four groups of goats were used: (1) sinus rhythm (SR), (2) 4 months’ AF (4 mo AF), (3) 2 months’ SR after 4 mo AF (2 mo post-AF), and (4) 4 months’ SR after 4 mo AF (4 mo post-AF). Atria were characterized electrophysiologically, (ultra)structure was studied by light and electron microscopy, and structural and gap-junctional protein expression was studied by immunohistochemistry or Western blotting. The atrial effective refractory period had completely returned to normal values 2 mo post-AF. Induced AF episodes still lasted for minutes at 2 and 4 mo post-AF, compared with seconds in the SR group. Structural abnormalities were still present at 2 and 4 mo post-AF, although to a lesser extent. The increased atrial myocyte diameter was back to normal at 4 mo post-AF. The number of myocytes with severe myolysis had almost normalized 4 mo post-AF, whereas myocytes with mild myolysis remained significantly increased. Extracellular matrix area fraction after 4 mo AF was similar to SR. However, the extracellular matrix fraction per myocyte had increased after 4 mo AF and remained higher post-AF. Changes in expression of structural proteins were partially restored post-AF. The reduction of connexin 40 that was observed during AF was completely reversed at 4 mo post-AF.

Conclusions—Recovery from structural remodeling after 4 mo AF is a slow process and is still incomplete 4 mo post-AF. Several months post-AF, the duration of AF episodes is still prolonged (minutes). (Circulation. 2003;107:2051-2058.)

Key Words: atrium ■ fibrillation ■ structure ■ gap junctions ■ remodeling

Atrial fibrillation (AF) has a progressive character, with paroxysmal AF often progressing to sustained AF. It has been established that chronic rapid atrial pacing in dogs and repeated induction of AF in goats results in the development of sustained AF (AF begets AF). The increasing stability of the repeated induction of AF in goats results in the development of a phenotype (dedifferentiation). Also, remodeling of gap junctions occurs during AF. In the goat, the expression of the gap junction protein connexin (Cx) 40 decreased and became distributed heterogeneously. The expression and distribution of Cx43 remained unaltered. Limited data are available on the reversal of structural and gap-junctional remodeling after prolonged AF. In the dog, Everett et al found no signs of recovery of atrial structural remodeling 2 weeks after cardioversion of AF, despite a complete reversion of electrical remodeling. In the present study, we investigated the reversal of structural and gap-junctional remodeling after cardioversion of 4 months of AF in the goat. Inducibility and stability of AF were also measured 2 and 4 months, respectively, after cardioversion.

Methods

Goat Model of Chronic Atrial Fibrillation

The goat model of AF has been described previously. Animal handling was carried out according to the Dutch Law on Animal Experimentation (WOD) and The European Directive for Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. Under general anesthesia, an Itrel pacemaker (Medtronic) was implanted subcutaneously in the neck, and a bipolar screw-in electrode was inserted through the jugular vein in the right atrium. AF was maintained by burst stimulation. Four groups of 6 goats (62±13 kg) were used. One group was kept in SR, 1 group was kept in AF for 4 months by burst pacing (4 mo AF), 1 group was kept in...
SR for 2 months after 4 months’ AF (2 mo post-AF), and 1 group was kept in SR for 4 months after 4 months’ AF (4 mo post-AF). In the post-AF groups, SR was restored either pharmacologically by the class lc drug cibenzoline (half-life 8 to 12 hours and therefore not expected to influence structural remodeling), or in case of failure of pharmacological cardioversion, by internal electrical cardioversion.12,13

At the end of the experimental period, the goats were anesthetized and the chest was opened. Electrophysiological measurements were taken with electrodes sutured to the epicardial surface of both atria. The extracellular matrix (ECM) surface area fraction was determined by morphometry, and was expressed relative to the myocyte surface area \( \left( \frac{\pi \cdot \text{diameter}^2}{4} \times \% \text{ECM area} \right) \).

**Extracellular Matrix**
The extracellular matrix (ECM) surface area fraction was determined by morphometry and was expressed relative to the myocyte surface area \( \left( \frac{\pi \cdot \text{diameter}^2}{4} \times \% \text{ECM area} \right) \).

**Immunohistochemistry and Western Blotting**
The organization and expression of structural proteins and connexins was studied by immunofluorescence. The immunoperoxidase method was used for staining of α-smooth muscle actin (SMA).

**Statistical Analysis**
Because most of the data were not normally distributed, groups were compared by a Kruskal-Wallis 1-way ANOVA by ranks, followed by pairwise Wilcoxon-Mann-Whitney rank-sum tests. Two-sided probability values were adjusted for multiple comparisons with the Bonferroni-Holm procedure. Analyses were performed with SAS V8.2 software.

**Results**

**Electrophysiology**
To check for complete reversion of electrical remodeling at 2 and 4 mo post-AF, the AERP was measured at pacing intervals of 400 and 200 ms. AERP400 was 115±7 ms at 2 mo and 121±13 ms at 4 mo post-AF, not significantly different from SR (130±15 ms) (Table 1, Figure 1A). Similar data were obtained for AERP200. The AF cycle length, which

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**TABLE 1. Recovery of Electrophysiology**

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>4 mo AF</th>
<th>2 mo Post-AF</th>
<th>4 mo Post-AF</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>AERP400, ms</td>
<td>130±15</td>
<td>115±7</td>
<td>121±13</td>
<td></td>
<td>0.130</td>
</tr>
<tr>
<td>AERP200, ms</td>
<td>134±26</td>
<td>118±20</td>
<td>123±3</td>
<td></td>
<td>0.545</td>
</tr>
<tr>
<td>AF cycle length, ms</td>
<td>124±43</td>
<td>87±10*</td>
<td>128±6†</td>
<td>143±16†</td>
<td>0.301</td>
</tr>
<tr>
<td>Median AF duration, s</td>
<td>6.5±4.2</td>
<td>∞*</td>
<td>127±189†</td>
<td>36±32†</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean AF duration, s</td>
<td>6.8±4.6</td>
<td>∞*</td>
<td>783±1379†</td>
<td>1005±2163†</td>
<td>0.001</td>
</tr>
<tr>
<td>AF duration &gt;1 min, %</td>
<td>0.65±1.59</td>
<td>∞*</td>
<td>46.2±41.2†</td>
<td>31.8±37.8†</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean±SD. *P<0.05 vs SR; †P<0.05 vs 4 mo AF (Bonferroni-Holm procedure).
‡P value of Kruskal-Wallis 1-way ANOVA by ranks.
shortened during AF, was comparable to that in SR at 2 and 4 mo post-AF (Table 1, Figure 1B). AF episodes immediately after cardioversion after 4 mo AF were very stable and not self-terminating. Episodes of induced AF lasted longer in the post-AF groups than in SR [median duration, 127 seconds (2 mo post-AF) and 36 seconds (4 mo post-AF) versus 6.5 seconds (SR)] (Table 1, Figure 1C). The variability in duration of AF episodes was larger post-AF. Although episodes as short as a few seconds were registered, the proportion of AF episodes lasting >1 minute was 38% at 2 mo post-AF and 18% at 4 mo post-AF compared with only 0.7% in SR (Figure 1D).

**Morphology**

**Ultrastructure**

Atrial myocytes from goats in SR had sarcomeres that were distributed regularly throughout the cytoplasm, with rows of uniformly sized mitochondria between them and nuclei with clustered heterochromatin (Figure 2A). During sustained AF (4 mo), a significant number of myocytes was affected by myolysis. No degenerative or atrophic changes were present; the size of the atrial myocytes was even increased. In areas depleted of sarcomeres, mitochondria were small and elongated, and remnants of sarcoplasmic reticulum were often seen (Figure 2B). Two and 4 mo post-AF, abnormal small mitochondria and not regularly organized sarcoplasmic reticulum visible as loose membrane structures were still present (Figure 2, C and D). In contrast, the nuclear heterochromatin, which became uniformly dispersed throughout the nucleoplasm during AF, had normalized again 4 mo post-AF (Figure 2D).

**Light Microscopy**

**Myolysis**

After 4 mo AF, considerable numbers of atrial myocytes were typically affected by myolysis (mild+/severe) and showed glycogen accumulation (Table 2, Figure 3B). At 2 mo post-AF, the number of myolytic myocytes was still high, although the incidence of cells with severe myolysis had decreased significantly (Table 2, Figure 3C). At 4 mo post-AF, the number of both severely and mildly affected myocytes had decreased, although they were still higher than in SR (Table 2, Figure 3D).

**Atrial Myocyte Diameter**

The myocyte diameter, which increased during AF (Table 2, Figure 3B), had decreased post-AF. Whereas at 2 mo post-AF, values were still higher than in SR, at 4 mo post-AF, their size had normalized (Table 2, Figure 3D).

**Extracellular Matrix**

At 4 mo AF, the ECM surface area fraction was increased slightly (Table 2, Figure 3F). At 2 mo and 4 mo post-AF, these values were still increased slightly compared with SR (Figure 3, G and H). Because atrial myocytes were larger during AF, the relative area fraction of ECM per myocyte had increased (Table 2, Figure 3G). At 2 mo post-AF, a further increase in ECM surface area per myocyte was seen, whereas this ratio decreased again at 4 mo post-AF, although it was still higher than in SR (Figure 3H).

**Structural Proteins**

**Cardiotin**

In contrast to SR, cardiotin staining was absent during AF and recovered completely early after AF. The distribution of cardiotin in arrays parallel to the sarcomeres was already restored at 2 mo post-AF (Table 3, Figure 4A).

**Titin**

In goats in SR, the A-I junctional part of titin showed clear cross-striated staining patterns. The overall labeling intensity was reduced significantly at 4 mo AF; only a punctate, less intense staining remained (Figure 4B, Table 3). At 2 mo post-AF, the staining pattern of titin partially recovered. At 4 mo post-AF, a further increase in intensity and amount of cross-striations was observed, although it was still lower than in SR.
Atrial myocytes from goats in SR lack α-SMA staining (Figure 4C, Table 3). After 4 mo AF, a substantial portion of the myocytes displayed an intense staining, whereas the remaining myocytes were diffusely stained for α-SMA. Post-AF, the intensity of α-SMA staining decreased, although this was seen as short, transversely oriented lines and in IDs. In longitudinally sectioned myocytes, Cx40 and Cx43 showed a homogeneous distribution in goat atria during SR (Figure 5A). Both gap junctions colocalized that in SR. Gap Junctions

Cx40 and Cx43 showed a homogeneous distribution in goat atria during SR (Figure 5A). Both gap junctions colocalized to a large extent in IDs. In longitudinally sectioned myocytes, this was seen as short, transversely oriented lines and in disks (IDs), desmoplakin, desmoglein, and N-cadherin staining remained present, whereas desmin staining was absent at these sites (Figure 4D, Table 3). At 2 mo post-AF, desmin staining was observed again in a substantial portion of the IDs. A further recovery was observed 4 mo post-AF, when all IDs showed SR-like desmin staining, whereas the staining of desmoplakin, desmoglein, and N-cadherin was comparable to that in SR.
density. This resulted in clearly decreased levels of Cx40 in areas with a normal density or almost devoid of Cx40 next to areas with a significantly lower density, showing areas with a significantly lower AF, the Cx40 distribution pattern in both atria had become more heterogeneous, creating a situation in support of microreentry leading to sustained AF. The complete absence of the gap junction protein Cx40 in mouse atrium has been shown to significantly increase its susceptibility to arrhythmias. In goat atrium, AF was associated with changes in distribution and expression of Cx40. The microheterogeneity in the goat atrium, AF was associated with changes in distribution and expression of Cx40. The microheterogeneity in the distribution of gap junctions (small clusters of well-coupled atrial myocytes amid clusters lacking Cx40) creates a situation in support of microreentry leading to sustained AF. However, the observed changes did not seem to have a significant impact on overall intra-atrial conduction velocity during SR. During chronic AF, atrial activation had an increasing complexity. In modeling studies, it was shown that conduction velocity is only moderately sensitive to a

gap junctions, whereas the expression of Cx43 was not changed (Figure 5A, Table 3). Already at 2 mo post-AF, Cx40 levels had normalized completely, and the distribution was primarily homogeneous again. The degree of colocalization of Cx40 and Cx43 remained somewhat lower than during SR. By confocal microscopy-based image analysis, the average Cx40/Cx43 fluorescence ratios were determined from 10 areas in 5 or 6 goats per group (Figure 5A, bar graphs). For each animal in the 4 mo AF group, these ratios were significantly lower than in the SR group. At 2 mo post-AF, ratios were normalized and on average even exceeded those in the SR group, although the variation was high. Also, Western blotting showed that the level of Cx40 proteins was lower after 4 mo AF than during normal SR. At 2 or 4 mo post-AF, Cx40 protein levels recovered, whereas those of Cx43 had remained stable (Figure 5B). The histograms show the Cx40/Cx43 protein ratios on the basis of density measurements of gel bands. Values were reduced up to 60% after 4 mo of AF and during 4 mo after cardioversion, they recovered to >80% of the baseline value.

Discussion

In the present study, it was shown that recovery from structural remodeling is a very slow process. The electrophysiological impact of structural remodeling on AF was also tested. The AF cycle length and atrial refractory period were completely reversed at 2 mo post-AF, in analogy with previous studies that showed that electrical remodeling was reversible within a few days. The median duration of induced AF episodes increased from seconds during SR to minutes post-AF but did not last for hours, as seen after a few weeks of AF. In goats, repeated induction of AF by burst pacing leads to the development of persistent AF after 2 to 3 weeks. The electrical remodeling occurring within the first few days is responsible for the increasing stability of AF but cannot explain the development of persistent AF. Therefore, a second factor with a slower time course must be involved. The development of persistent AF occurs within the same time domain as structural and gap-junctional remodeling occur.

Possible Contributions of Structural and Gap-Junctional Remodeling to Stabilize AF

It is not easy to understand how certain changes in cellular structure, such as increased cell size, glycogen accumulation, and different expression of structural proteins, can play a role in the perpetuation of AF. The complete absence of the gap junction protein Cx40 in mouse atrium has been shown to significantly increase its susceptibility to arrhythmias. In goat atrium, AF was associated with changes in distribution and expression of Cx40. The microheterogeneity in the distribution of gap junctions (small clusters of well-coupled atrial myocytes amid clusters lacking Cx40) creates a situation in support of microreentry leading to sustained AF. However, the observed changes did not seem to have a significant impact on overall intra-atrial conduction velocity during SR. During chronic AF, atrial activation had an increasing complexity. In modeling studies, it was shown that conduction velocity is only moderately sensitive to a

Table 3. Recovery of Structural and Gap-Junctional Proteins

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>4 mo AF</th>
<th>2 mo Post-AF</th>
<th>4 mo Post-AF</th>
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<tbody>
<tr>
<td>Cardiotin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>LAA</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Titin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>+++</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LAA</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-SMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LAA</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Desmin at intercalated disk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>+++</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>LAA</td>
<td>+</td>
<td>–</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Connexin 40</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>+++</td>
<td>–</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>LAA</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Connexin 43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAA</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>LAA</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

RAA indicates right atrial appendage; LAA, left atrial appendage.
reduction in gap-junctional conductance and that cell dimensions may be as important. This means that an AF-induced cellular volume increase, as observed in the goat, can theoretically compensate for changes in overall conduction velocity as result of gap-juncational remodeling. Recovery of Cx40 gap-junctional remodeling was almost complete at 2 mo post-AF. This suggests that although gap-junctional remodeling plays a role in stabilizing the arrhythmia after weeks of AF, it is not responsible for the slightly prolonged duration of AF episodes (minutes) after 2 and 4 mo post-AF.

The small increase in ECM detected might result in inhomogeneous conduction. The presence of collagen septa will result in electrical uncoupling of parallel-oriented fibers. Spach et al showed that anisotropic electrical properties of atrial muscle produces complex pathways of propagation spread. Increased collagen levels or a reduction in sarcomeres results in areas with abnormally slow conduction, block, and reentry, which in turn stabilize AF. In dogs with ventricular pacing–induced congestive heart failure, a substrate (increased ECM amount) that can support prolonged AF remained after a 5-week recovery period.

Atrial dilatation resulting from the loss of atrial contractility, influenced by structural remodeling, will also increase the number of wavelets. Some studies showed that atrial enlargement is positively correlated with the incidence and stability of AF. Boyden et al, studying the relationship between susceptibility to arrhythmias, dilatation, and structural remodeling, found that dogs with valvular disease had hypertro-

Figure 4. Immunohistochemistry. A, Cardiotin (green) and DAPI nuclear staining (blue). B, Titin (red) and DAPI staining. C, \(\alpha\)-SMA (red-brown) and hematoxylin nuclear staining (blue). Vascular \(\alpha\)-SMA staining is always present (arrowhead). D, Desmin (red) and DAPI. Intensified desmin staining at the ID (arrowhead). m indicates months.
phied myocytes with structural alterations such as myolysis and glycogen accumulation and some increase in connective tissue between the cells. The action potential duration was not altered in these dogs, but the proportion of atrial arrhythmias lasting >10 minutes was increased significantly. Regional differences in wall thickness, resulting in inhomogeneous wall stress, will add further to the increased heterogeneity in conduction. The exact contribution of the different factors of structural remodeling in stabilizing AF is still unknown.

Why Is Recovery From Structural Remodeling Slow?

The decreased expression of Cx40 was reversed at 2 mo post-AF. In cells that express GFP-tagged Cx43, the association of connexins with gap junctions was shown to be highly dynamic.26 Because the half-life of Cx-43 is rather short (1.3 hours),27 recovery from gap-junctional remodeling might be a relatively fast process.

Recovery from structural remodeling in general appeared to be a slow process. It has been known for many years that regression of a hypertrophic response, especially of the left ventricle, may take several months.28 Hypertrophic remodeling of myocytes is characterized by an increased synthesis of sarcomeres, whereas during later stages, myolysis and elevated levels of connective tissue might also be present.29 Regression from hypertrophy can occur if the volume or pressure overload did not exist too long, because the point of no return should not be reached. Hypertrophic myocytes can decrease their cell size by removal of sarcomeres.30 Ultrastructural changes such as the appearance of small mitochondria and loss of alignment of sarcomeres reverses after several months.31 In case of an atrophic response, after contractile arrest, the recovery of myofilament structure occurs within 2 weeks.32 In the canine model of AF, Everett et al10 did not observe reversion of structural atrial remodeling by 2 weeks of SR after 2 mo AF despite full recovery from electrical remodeling, possibly because of the remaining mitral valve insufficiency after cardioversion. The reason for the slow recovery of contractile material in our study is unknown. A contributing factor might be the reduced atrial contractility after long-term AF resulting in an increased stretch that promotes Ca²⁺ overload, which subsequently sustains proteolysis. In addition, stretch might give rise to enhanced ECM formation.

It was recently shown that in chronic heart failure, induced atrial ECM remodeling was not reversed after 5 weeks. In our study, only a small reduction in ECM fraction occurs 4 mo post-AF. Increased stretch caused by dilatation of the atrial wall might stimulate ECM formation or reduce resynthesis of myofibrillar proteins. The mechanisms controlling protein synthesis and degradation in myocytes in response to different stimuli are not well understood.

Concluding Remarks

The present study showed that recovery from structural remodeling after months of AF is a very slow process. At 4 mo post-AF, several structural changes were still present to a significant degree, whereas gap-junctional remodeling was shown to be completely reversed. Longer post-AF periods will be necessary to find out whether a full recovery from structural remodeling is possible. For studying the exact timing of gap-junctional remodeling, earlier time points will be necessary. Several months after AF, the duration of AF episodes is still prolonged (minutes). This is not because of electrical remodeling, because the AERP was shown to be
back to normal values. Nor was it because of gap-junctional remodeling, because this process was also completely reversed. Previous studies indicated that the overall atrial conduction velocity did not change because of sustained AF.\textsuperscript{3,4} Electrophysiological data from the present study are limited. The tissue reaction in response to the chronically implanted electrodes required for serial electrophysiological testing would have jeopardized the primary goal of our study, ie, the accurate characterization of AF-induced structural remodeling. To identify the effects of the altered myocardial structure on the propagation of action potentials and stability of AF, further studies will be required. Special attention should be given to the increased connective tissue content and atrial dilatation. Similar structural changes as described here have been reported for patients with AF and other underlying heart diseases.\textsuperscript{5,6} Some of these are far more extensive, eg, fibrosis and cellular degeneration, which is probably related to age and/or associated heart disease. Hence, the recovery from structural remodeling in patients, if reversible at all, might take even longer. If structural changes are still present, new episodes of AF are expected to lead more readily to sustained AF.

Acknowledgments
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References
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